Reproductive steroids are detectable in the faeces of dugongs

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ABSTRACT

Reproductive steroid (oestradiol and testosterone) levels were measured in the faeces of ten wild dugongs (*Dugong dugon*) from Moreton Bay Australia, using radioimmunoassay (RIA). All faecal samples, including from both genders and from both immature and mature dugongs contained detectable levels of oestradiol and testosterone. Faecal testosterone levels were high relative to oestradiol concentrations in all samples, regardless of gender. The highest levels of testosterone occurred in adults (body lengths ≥250 cm), in both males (1146.7 ng g^{-1} dry weight faeces) and females (691.1 ng g^{-1} dry weight faeces). The highest oestradiol levels were recorded in adult females (37.2 ng g^{-1} dry weight faeces). In mature dugongs, the ratio of testosterone to oestradiol was higher in adult males than in females, suggesting that this ratio may be a better indicator of sex than levels of individual steroids. This study represents the first step in an investigation into assessing the reproductive status of free-ranging dugongs.

Key words: Gonadal steroids, faeces, dugong, reproduction, hormones, oestradiol, testosterone, *Dugong dugon*

Introduction

The relatively non-invasive approach of estimating reproductive hormones in faecal samples is well established for monitoring reproductive function in a variety of captive and free-ranging mammalian species (e.g. Schwarzenberger *et al* 1993, 1996; Heistermann *et al* 1995; Kirkpatrick *et al* 1996; Berkeley *et al* 1997; Brown and Wildt 1997; Larkin *et al* 2005). Gonadal steroid concentrations in faeces reflect gonadal function (Brown and Wildt 1997) and exhibit a similar pattern to those concentrations in blood plasma but usually have a lag time of between twelve hours and two days depending on the mammalian species (Schwarzenberger *et al* 1996). Moreover, faecal steroid estimations have two major advantages over measurements of steroid concentrations in peripheral blood: (i) they are much less prone to sampling artefacts than are peripheral blood concentrations in wild mammals; and (ii) in any case, faecal concentrations present an "integrated"' picture of hormone production rather than an "instantaneous" value: this often provides a more useful estimate for observing seasonal effects. Faecal oestrogen and progesterone levels have been used as reliable indicators of ovulation, pregnancy and parturition in several ungulate, primate and carnivore species; faecal testosterone concentrations have been used widely to observe reproductive seasonality in male mammals.

This paper reports on a study examining the feasibility of measuring faecal steroids (oestradiol and testosterone) to assess reproductive activity in free-ranging Dugongs, *Dugong dugon*. The first mark-recapture study of free-ranging *D. dugon* in the world has been established in Moreton Bay, south-east Queensland, Australia (Lanyon *et al*. 2002). In addition to tagging individual *D. dugon* for long-term identification, each animal has been measured and sexed. Restraining a wild *D. dugon* in an open water situation is not always conducive to more invasive sampling such as the taking of blood to measure reproductive hormone levels. However, it is not uncommon for faecal material to be produced by restrained animals, and this provides the opportunity to measure the output of reproductive steroids.

Methods

Faecal samples were collected from ten *D. dugon* in Moreton Bay. Seven of the 10 *D. dugon* were wild-caught using the dugong rodeo technique described in Lanyon *et al*. (2002) and faeces were collected as they were eliminated by the restrained animal. Gender and body length (snout to fluke notch), and time and location of capture were recorded. These *D. dugon* included animals of both sexes, and both immatures and adults where sexual maturity of male and female *D. dugon*, in tropical regions at least, is achieved at body lengths of less than 2.2m and greater than 2.5m respectively (Marsh *et al* 1984b; Marsh 1995). The remaining faecal samples were taken from the distal colons of three freshly-dead male *D. dugon* that were killed by boat-strike, within 24h of death. Faeces were sub-sampled, frozen upon collection and stored at -20°C until analysed.

Gonadal steroids were measured by radioimmunoassay (RIA). Hormones were extracted from faecal samples using both water and ethanol extraction to maximise steroid return. Water extraction involved measuring approximately one gram of faecal material in a plastic vial with the equivalent mass of distilled water. Each sample was vortexed for 1 minute, centrifuged for 10 minutes and the supernatant retained. The ethanol extraction method was a modified version of Brown *et al.* (1994). Five ml of 90% ethanol was added to the pellet (following water extraction), vortexed for 1 minute, boiled for 20 minutes, centrifuged for 10 minutes at 500 x g and the supernatant retained. This procedure was repeated to yield a third supernatant, which were then all combined. The combined supernatant was boiled dry overnight. One ml of 100% methanol was then added and the sample vortexed for 1 minute, placed in an ultrasonic glass cleaner for 30 seconds, then vortexed for another 15 seconds. The resultant solutions were used in RIAs.

The oestradiol (E2) and testosterone (T) RIAs were conducted using DiaSorin ESTR-CTK-4 and TEST-CTK kit (ref no. P3093) assay kits respectively. The principle of these assays is based on competition between radio-actively labelled steroid and steroid contained in the samples for a fixed and limited number of binding sites on the inside coating of test tubes. After incubation, the amount of labelled steroid bound to antibody on the tube walls is inversely related to the concentration of unlabelled steroid in the samples. These kits have analytical sensitivities of 4pg/ml for E2 and 0.02ng/ml for T. The antibodies are highly specific for the steroids, with low cross-reactivities with other steroids. Standard procedures were followed to produce tubes coated with the sample steroid antibodies. Radioactivity (^{125}I-labelled testosterone) of the tubes was measured, using an LKB Wallac 1277 Gammamaster gamma counter with a run protocol of 1 minute. Each standard calibrator, control and sample was run in duplicate. Concentrations of steroids in the samples were determined against standard curves produced for each steroid.

Results

All *D. dugon* faecal samples had detectable levels of oestradiol and testosterone. Oestradiol ranged from a lowest recorded level of 3.7 ng g^{-1} dry weight faeces in an immature male to a highest recorded level of 37.2 ng g^{-1} dry weight faeces in an adult female, however, comparably high levels (37.1 ng g^{-1} dry weight faeces) were also recorded in a male (immature, 201cm) (Figure 1a). The highest levels of oestradiol in females were in the two adults (body length >250cm); the third female (241cm) with lower oestradiol levels may be an immature or adult (after Marsh *et al* 1984b). There were no faeces sampled from female *D. dugon* less than 241 cm body length. There was no appreciable difference in oestradiol levels between immature and adult males (Figure 1a).

Testosterone levels ranged from 152.2 to 1146.7 ng g^{-1} dry weight faeces (Figure 1b). In all faecal samples, measurable testosterone concentrations were higher than oestradiol levels. In males, the highest levels recorded were generally in adults (>250cm body length) and the lowest in immatures/calves (Figure 1b). In adult females, testosterone levels ranged from 347.9- 691.1 ng g^{-1} dry weight faeces, falling within the range of adult males. Testosterone concentrations were higher in the faeces than the concentrations in the standards in 10 of the 11 samples.

The ratios of testosterone to oestradiol concentrations (T:E2) in faeces of *D. dugon* were highest in mature males and lowest in immature animals. Of the mature *D. dugon* (>250cm body length), males had markedly higher T:E2 (61:1, 113:1 & 117:1) than females (14:1 & 19:1) (Figure 2). There was no clear trend in T:E2 in *D. dugon* of body length <250cm.

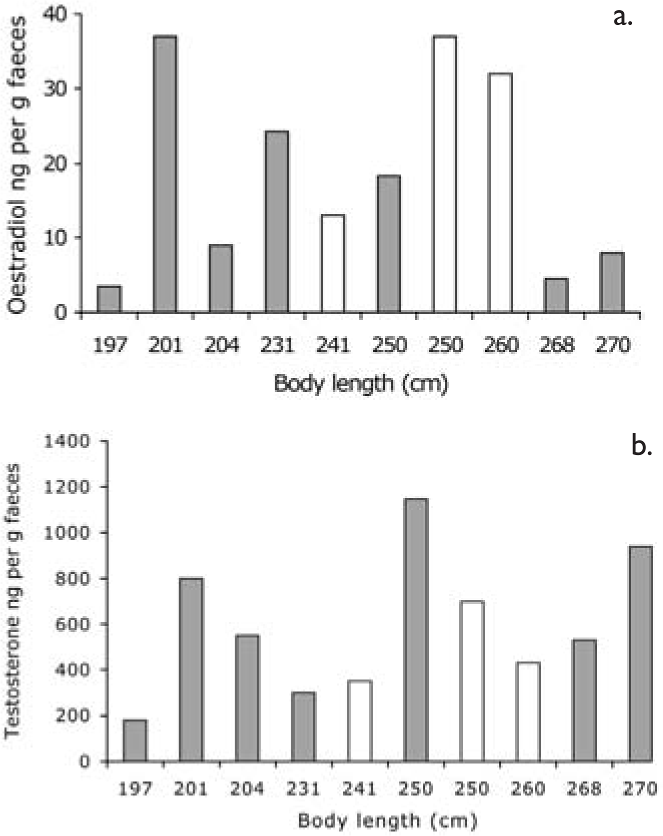

Figure 1: Concentrations of **a.** Oestradiol (ng g^{-1} dry weight faeces) and **b.** Testosterone (ng g^{-1} dry weight faeces) in *D. dugon* of known sex and body length. Females are open bars; Males are solid bars. Body length > 250cm = adults; body length <220cm = immature; body length >220cm but <250cm = immature or adult (Marsh **et al** 1984b).

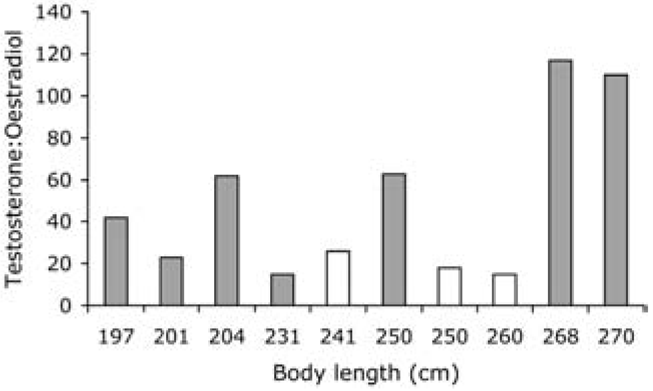

Figure 2. Testosterone to Oestradiol (T:E2) ratio in faeces of ***D. dugon*** of known sex and body length. Females are open bars; Males are solid bars. Body length > 250cm = adults; body length <220cm = immature; body length >220cm but <250cm = immature or adult.

Discussion

Oestradiol and testosterone were detected in the faeces of all *D. dugon* sampled including in both genders and mature and immature animals. In particular, faecal testosterone concentrations were high, relative to both sample oestradiol concentrations and to concentrations of testosterone in the assay standards. These high testosterone levels may reflect naturally-occurring levels in faeces of *D. dugon*, considering that a faecal sample reflects hormone secretions occurring hours to days before excretion (Lasley and Kirkpatrick 1991, Vincent and Mitchell 1992, New *et al* 1998), i.e. a temporal accumulation of hormone. Alternatively there may be a component of faeces that is denaturing the assay antibodies or blocking the antibody binding sites. Either of these events would lead to less bound hormone being detected and an overestimation of the free testosterone in samples. Such an artefact could explain why the two female adult *D. dugon* did not have higher concentrations of oestradiol than testosterone in their faeces, as may have been expected. This anomaly could presumably be resolved through subsequent trialling of the efficiency of hormone extraction after adding known amounts of testosterone to faecal samples. However, faecal testosterone levels measured in this study fall within the range of levels measured in Florida manatees, a relative of the dugong, using a similar RIA assay. Faecal testosterone levels in manatees range from 120.8 to 36,240 ng g^{-1} dry weight faeces in males, and from 120.8 to 5,919 ng g^{-1} dry weight faeces in females (Larkin *et al* 2005).

Faecal steroid concentration could not be analysed quantitatively with regard to gender and maturity of *D. dugon* due to small and unequal sample sizes of animals over a broad size/age range. If *D. dugon* with body lengths >250cm are considered mature adults, and smaller animals as probable immatures (after Marsh *et al* 1984b), only three mature males and two mature females were included in the adult data set. Based on these limited specimens, levels of faecal testosterone were highest in mature male *D. dugon*, with lower levels in immature males. Levels of testosterone in the two adult females fell within the range for adult males, and were higher than in the immature female. With respect to faecal oestradiol, the highest levels were recorded in the two adult females, with lower levels in the smaller female. There was no trend of increasing oestradiol with maturity in males; in fact, higher levels were recorded in immature rather than mature males. In mature *D. dugon*, the ratio of testosterone to oestradiol (T:E2) was markedly higher in adult males than in adult females and this may be a better indicator of the mature sexes than levels of individual steroids.

Since neither male nor female *D. dugong* are continually in breeding condition (Marsh *et al* 1984b), steroid level (and sexual activity) is likely to vary temporally. *D. dugong* in tropical regions of Australia show a diffusely seasonal breeding cycle, with the peak of male sexual activity (assessed by maturity and function of testes) occurring between June and January, with female gonadal activity overlapping this period (Marsh 1995). Further, there is some evidence that fully mature male *D. dugong* may enter sterile cycles on a long term or permanent basis (Marsh *et al* 1984a). Since dugongs in this study were sampled in more southern sub-tropical Moreton Bay, we might expect a more pronounced seasonality with a shorter, more defined breeding period. It is possible that some of the variation in steroid levels recorded here is related to seasonality of gonadal activity, e.g., the three male dugongs in which oestradiol levels were highest were all sampled in August. The influence of season will be examined as more samples become available.

The validity of such a non-invasive technique relies on the assumption that the steroid metabolite concentrations detected in the faeces accurately reflect concentrations in the blood (Hirschenhauser *et al* 2000). In this study, faecal samples have been collected from free-ranging dugongs on an opportunistic basis with a view to sequentially (i) analyzing them to see if detectable levels of steroids are present, (ii) calibrating faecal steroids against blood plasma steroids in a captive situation and (iii) when available, using faecal steroids to obtaining seasonal life history data for individual dugongs in a wild population. The next step in this investigation will involve calibration of faecal and blood hormone levels. We also plan to analyse faecal oestrogen and progesterone levels to assess whether these could be used in concert as reliable indicators of ovulation, pregnancy and parturition in free-ranging *D. dugon*.

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